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Platelet aggregation and antibacterial effects of an L-amino acid oxidase purified from *Bothrops alternatus* snake venom

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Abstract—The isolation and biochemical/enzymatic characterization of an L-amino acid oxidase, Balt-LAAO-I, from *Bothrops alternatus* snake venom, is described. Balt-LAAO-I is an acidic glycoprotein, p$I \sim 5.37$, homodimeric, $M_r \sim 123,000$, whose N-terminal sequence is ADVRNPLE EFRETDYEVL. It displays a high specificity toward hydrophobic and basic amino acids, while deglycosylation does not alter its enzymatic activity. Balt-LAAO-I induces platelet aggregation and shows bactericidal activity against *Escherichia coli* and *Staphylococcus aureus*. In addition, this enzyme is slightly hemorrhagic and induces edema in the mouse paw. Balt-LAAO-I is a multifunctional enzyme with promising relevant biotechnological and medical applications.

1. Introduction

L-Amino acid oxidases (LAAO, EC 1.4.3.2) are flavoenzymes, which catalyze the stereospecific oxidative deamination of an L-amino acid substrate to a corresponding α-ketoacid with the production of hydrogen peroxide and ammonia, via an imino acid intermediate.[1] These enzymes are widely distributed in many different organism such as bacteria, fungi, green algae, and venomous snakes[2–7] and are involved in the utilization of nitrogen sources.

LAAOs are present at significantly high concentrations in venoms where they are postulated to be toxins.[7–9] Although the action mode of snake venom LAAOs is not known, it has been shown that the enzymes form *Crotalus adamanteus* and *Crotalus atrox* can associate specifically with mammalian endothelial cells.[10] Before 1990s, the studies on snake venom L-amino acid oxidases dealt mainly with their enzymatic and physiochemical properties such as reaction mechanism and inactivation by pH changes or freezing.[11–13]

Recently, snake venom LAAOs have become an interesting object for pharmacological, as well as structural and molecular biology studies. Although little is known about their primary and tertiary structures, the cDNA sequences of two LAAOs from rattlesnakes (*C. atrox* and *adamanteus*) were determined and the possible FAD-binding and N-glycosylation sites were established.[14,15] The structure and substrate trajectory into the active site of the purified LAAO of *Calloselasma rhodostoma* snake venom have been also determined.[16] LAAOs have been characterized showing distinct molecular mass, substrate preference, apoptosis, cytotoxicity, hemolysis, platelet aggregation, induction of hemorrhage, edema, and bactericidal activities.[8,10,14,17–22]

Keywords: Snake venom; L-Amino acid oxidase; *Bothrops alternatus*; Bactericidal effect; Platelet aggregation; Biotechnological application
Abbreviations: LAAO, L-amino acid oxidase; Balt-LAAO-I, *Bothrops alternatus* L-amino acid oxidase-I; SV-LAAO, snake venom L-amino acid oxidase.

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The present investigation reports the isolation and biochemical characterization of an L-amino acid oxidase (Balt-LAAO-I) from *Bothrops alternatus* venom, with special reference to its platelet aggregation effect and bactericidal activity.

2. Results and discussion

2.1. Biochemical characterization of BaltLAAO-I

Balt-LAAO-I isolated by sequential purification steps as described (Fig. 1), showed to be highly purified by C-4 reverse phase HPLC as well as by SDS-PAGE (Fig. 2), representing approximately 1.0% of the total protein from *B. alternatus* venom. LAAOs from other snakes usually account for 1–9% of the total venom proteins,[23,24] except for *C. rhodostoma* venom, where LAAOs reaches 30%.[25]

Snake venom LAAOs (SV-LAAOs) represent an interesting bioactive model for enzymology, structural biology, and pharmacology. Recently, several SV-LAAOs have been purified and characterized, showing distinct M_r, substrate preferences, platelet interactions, hemorrhage induction, and apoptosis.[7]

Balt-LAAO-I was named so because it showed several isoforms, which were detected by isoelectric focusing (Fig. 1C). Different LAAO isoforms were also found in other snake venoms. This difference should reflect a post-translational glycosylation difference.[22] Balt-LAAO-I is a dimeric acidic glycoprotein with a p$I \sim 5.37$ and $M_r \sim 66,000$ for the monomer (SDS-PAGE) and 123,000 for the dimer (gel filtration) (Fig. 2C). PAGE for acidic proteins showed a single electrophoretic band fitting with the stained band for LAAO activity (Fig. 2D). LAAOs are usually homodimeric FAD-bound proteins of 110–150 kDa and pI from 4.4 to 8.12.[18,20,24,26,27] Only a single LAAO from *Bothrops* venom was so far isolated, namely, that from *B. moojeni*,[28] which is also dimeric and displays leishmanicide activity.

Balt-LAAO-I deglycosylation was confirmed by PAGE (data not shown). Sugars account for approximately

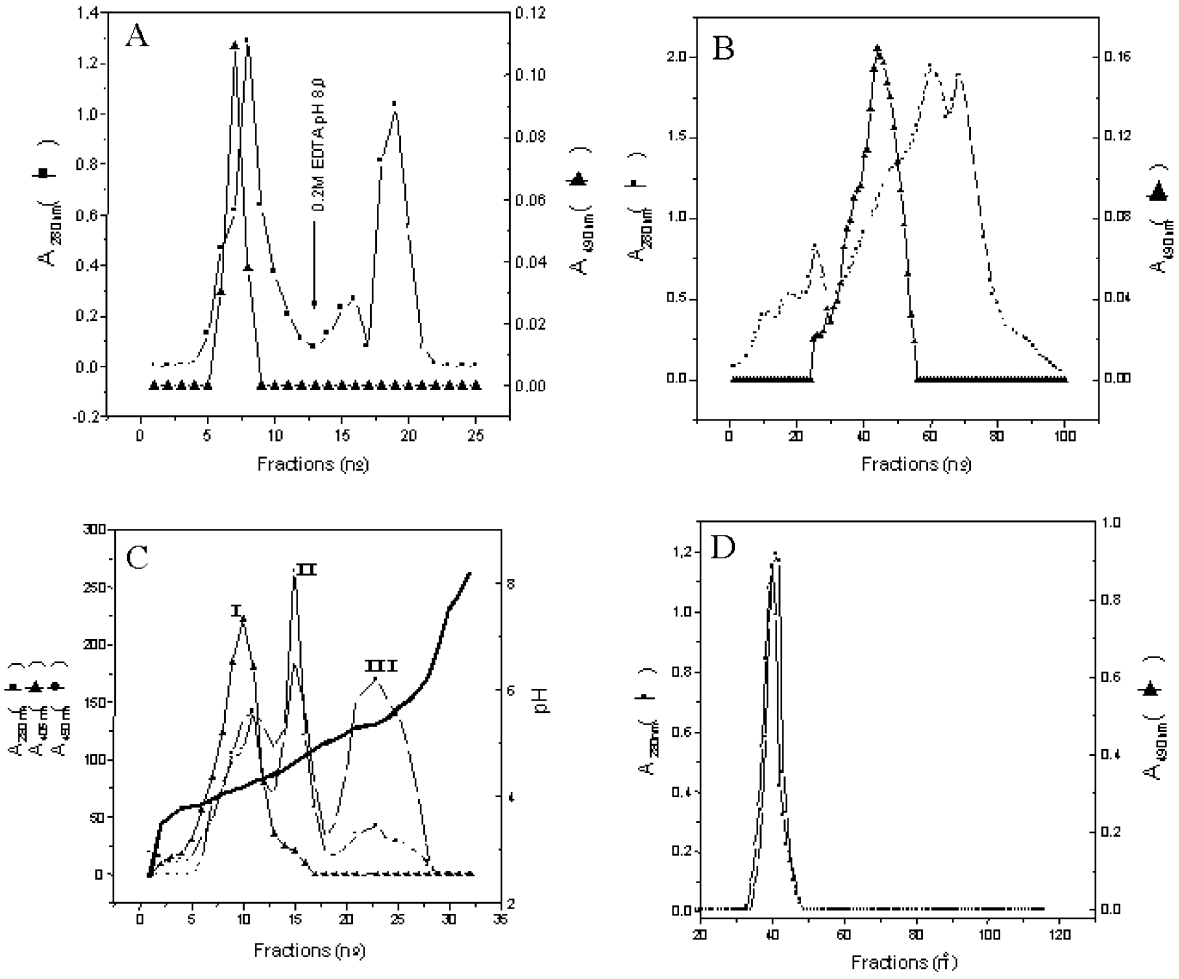

Figure 1. Sequential enzyme purification steps: (A) affinity chromatography of *B. alternatus* venom (1500 mg) on Sepharose-IDA, in 10 mM Tris/150 mM NaCl, flow rate: 1 mL/min, 7 mL/tube, 25 °C; (B) rechromatography of the active fraction on Phenyl-Sepharose in 50 mM Tris–HCl + 1.5 to 0 M $(NH_4)_2SO_4$; (C) isoelectric focusing of the still heterogeneous active fraction: (I) Balt-LAAO-p$I \sim 4.25$, (II) Balt-LAAO-p$I \sim 4.78$, and (III) Balt-LAAO-p$I \sim 5.37$; (D) p$I \sim 5.37$ isoform on Sephadex G-100 (0.9×180 cm) equilibrated with distilled water.

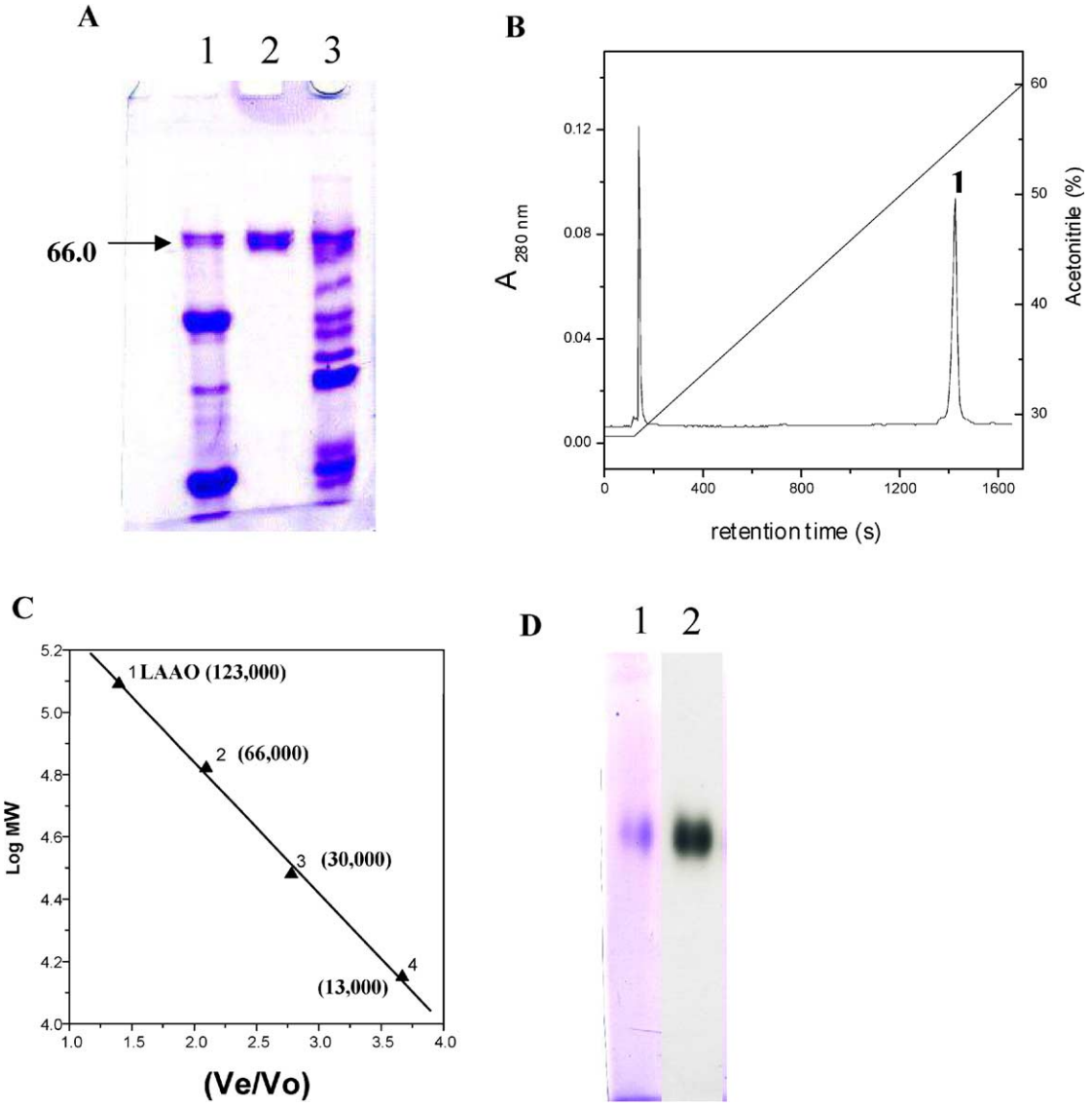

Figure 2. Electrophoretic analysis: (A) SDS-PAGE (12%) of the purified fractions: lanes (1) molecular weight markers; (2) final purified fraction of p*I* ~ 5.37 isoform (Balt-LAAO-I) from Sephadex G-100; (3) *B. alternatus* venom (B) chromatographic profile by HPLC; (C) gel filtration of Balt-LAAO-I dimer; (D) native PAGE (12%) of Balt-LAAO-I stained with coomassie blue (1) and for LAAO activity (2).

15% of its weight. The enzymatic activity was not lost after deglycosylation. The enzyme showed high affinity for Phe > Tyr > Met > Leu. For other amino acids, affinity was low or absent. After deglycosylation, the picture did not change, which suggests that the sugar portion of the molecule does not interfere with its enzymatic activity. L-Leu is the most used substrate to evaluate the enzymatic activity of purified SV-LAAOs but high catalytic activity was already detected against Phe, Trp, Met, Ile, and norleucine.[7] It was also reported that *Ophiophagus hannah* LAAO oxidizes L-Lys and L-Orn in addition to the above mentioned hydrophobic amino acids.[25]

SV-LAAOs have been extensively studied regarding their function, but structural studies were so far little explored, except for *C. rhodostoma* venom.[16] The amino acid composition of Balt-LAAO-I does not differ substantially from those of other reported venom LAAOs and is well characterized by the abundance of high contents of Asx and Glx and the presence of very low contents of Met and Trp residues.

The first 20 amino acid residues from Balt-LAAO-I were identified by automated Edman degradation analysis for additional evidence of purity and homology between different venom LAAOs (Table 1). Comparison of the N-terminal sequence of Balt-LAAO-I with the enzymes isolated from other snake venoms reported so far revealed close sequence homology in particular with *C. adamanteus* and *C. atrox* (80%), *Eristocophis macmahoni* (80%), *Trimeresurus flavoviridis* (78%), *C. rhodostoma* (70%), *Agkistrodon contortrix laticinctus* (69%), and *O. hannah* (45%). In the N-terminal sequences, at least nine amino acid residues (mainly glutamic acid) out of 20 were found to be fully conserved in all sequences, thus suggesting the presence of a highly conserved glutamic acid rich motif.

Table 1. Comparison of the N-terminal amino acid sequence of *B. alternatus* Balt-LAAO-I with others snake venom LAAOs

Snakes	N-Terminal sequences	Identity (%)	Reference
B. alternatus	ADVR**NPL-EE-FRETDYE**V-L	100	This study
Echis macmahoni	ADDK**NPL-EEAFREADYE**VFL	80	31
Cotralus atrox	AHDR**NPL-EECFRETDYEEFL**	80	14
C. adamanteus	AHDR**NPL-EECFRETDYEEFL**	80	15
T. flavoviridis	AHDR**NPL-EEYFRETDYEEFL**	20	
C. rhodostoma	ADDR**NPLAEE-FQENNYEEFL**	70	32
Agkistrodon c. laticinctus	ADSR**NPLEEE-FRETNYEEFL**	69	18
Naja n. kaouthia	DDRRS**PL-EECFQQNDYEEFL**	51	37
O. hannah	-SVI**N-L-EESFQEPEYENHL**	45	19
Consensus	**: * ** * : ** ***		

Completely conserved residues in all sequences are bolded and marked by asterisks. The gaps are inserted in the sequences in order to attain maximum homology.

2.2. Functional characterization of Balt-LAAO-I

Balt-LAAO-I induces dose dependent platelet aggregation (Fig. 3) either in platelet rich plasma (PRP) or in washed platelets (WRP) medium. It was able to cause platelet aggregation in rabbit and human plasma ($ED_{50} \sim 42\,\mu g/mL$). SV-LAAOs can inhibit or activate platelet aggregation.[7] Ali et al.[24] and Li et al.[8] showed that *E. macmahoni* and *O. hannah* LAAO induce platelet aggregation at 33 and 50 μg/mL, respectively. However, the action mechanisms of platelet aggregation by these enzymes should be better investigated.

Balt-LAAO-I also displays dose dependent bactericidal activity against both Gram positives and Gram negative bacteria (Fig. 4). Bactericidal effects were previously reported for LAAOs from *C. adamanteus*, *Pseudechis australis*, and *T. jerdonii* snake venoms.[22,29,30] Recently, it was demonstrated that *B. moojeni* LAAO caused death of the promastigote forms of different species of

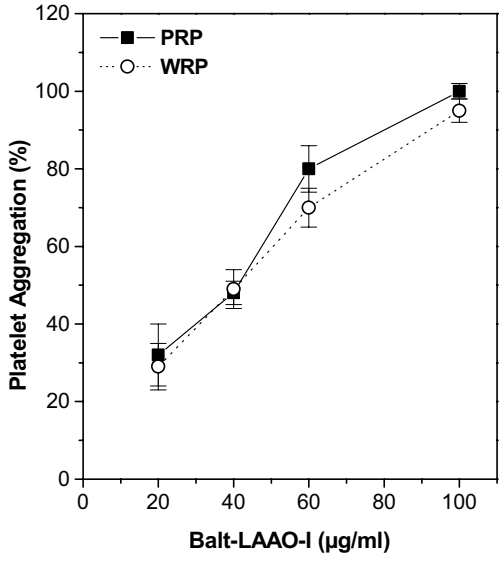

Figure 3. Effect of Balt-LAAO-I on platelet aggregation. Dose dependent platelet aggregation by Balt-LAAO-I in platelet rich plasma (PRP) and washed rabbit platelets (WRP) (4×10^5 cells/μL) at 37 °C. Control experiments were carried out using the platelet agonists alone (ADP or collagen). Results are reported as means ± SD ($n = 03$).

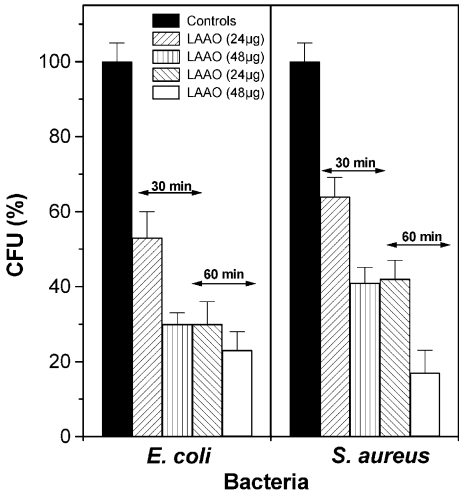

Figure 4. Bactericidal activity of Balt-LAAO-I upon *E. coli* and *S. aureus*. Different concentrations (24 and 48 μg) of Balt-LAAO-I were incubated with 4×10^5 CFU for 30 min and 1 h. Each bar represents the mean ± SD ($n = 03$).

Leishmania in vitro, consequently a potent therapeutic effect against leishmaniasis and other intracellular parasitic infestations.[28]

In addition, it induces edema in the mouse paw (results not shown). However, it is neither hemorrhagic nor myotoxic at doses up to 100 μg/animal (results not shown). Ali et al.[24] showed that *E. macmahoni* LAAO was able to induce edema in the mouse paw 1 h after administration of the toxin.

SV-LAAOs are therefore interesting multifunctional enzymes, not only for a better understanding of the ophidian envenomation mechanism, but also due to their biotechnological potential as model for therapeutic agents.

3. Experimental

3.1. Materials

Specimens of *B. alternatus* snakes were supplied by the biologist Luiz H. A. Pedrosa (FMRP-USP). Venom was

collected, vacuum desiccated and stored at 4 °C. All other reagents needed for chemical and biological characterization were acquired from Amersham Life Science Inc., Sigma Chem. Co., BioLab, GIBCO BRL or Mediatech.

3.2. Purification procedure

For the purification of LAAO-I, 1500 mg of desiccated *B. alternatus* venom (corresponding to 1.96×10^3 U) were dissolved in 100 mL of 10 mM Tris–HCl buffer, pH 8.8, containing 150 mM NaCl and applied on a Sepharose-IDA affinity column (3.0×68 cm), which was previously equilibrated and then eluted with the same buffer at a flow rate of 1 mL/min. One initial fraction, which was active upon L-Leu, was collected and kept for rechromatography under the same conditions. The remaining fractions were eluted after addition of 200 mM EDTA, pH 8.0, in the same buffer. The rechromatographed active fraction was vacuum concentrated to a volume of 5 mL, dialyzed against 50 mM Tris–HCl buffer, pH 8.1 containing 1.5 M ammonium sulfate and then submitted to a chromatography on Phenyl-Sepharose (1×34 cm) in the same buffer, at a flow rate of 0.8 mL/min. A decrescent concentration gradient from 1.5 M to 0 M ammonium sulfate in 50 mM Tris–HCl was applied to elute the fractions at room temperature. The active fraction was dialyzed against water and then vacuum concentrated to a volume of 5 mL. This sample was applied on a preparative isoelectric focusing device. Among the three active isoforms so obtained, the one with a p$I\sim5.37$ was collected, dialyzed against water, vacuum concentrated to 0.8 mL and finally applied on a Sephadex G-100 column (0.9×180 cm). The active fraction was collected and stored at 4 °C for future analysis.

For the purity assay, 1% of the sample was applied on a HPLC C4 reverse phase column (0.46×15 cm) equilibrated with 0.1% (v/v) trifluoracetic acid (TFA), followed by an acetonitrile concentration gradient from 28% to 60% (v/v) in 0.1% TFA for 32 min. The sample was assayed for purity by 11% (w/v) SDS-PAGE, as well as by PAGE for acidic proteins.

3.3. L-Amino acid oxidase assay

Activity of LAAO was determined in 0.1 M Tris–HCl buffer pH 7.2 and at 25 °C using an enzyme-coupled assay. In this assay, hydrogen peroxide generated by oxidative deamination of L-leucine by the enzyme was used by horseradish peroxidase to oxidize *o*-phenylenediamine to the colored product, which was spectrophotometrically monitored at $\lambda = 490$ nm; 20 mL assay mixture contained 10 μL horseradish peroxidase (1 mg/mL), 200 μL of an *o*-phenylenediamine solution (10 mg/mL methanol) and 20 mg of L-leucine. The reaction was carried out with 0.5 mL of the solution assay plus 0.5–5 μL of the LAAO solution and it was stopped by addition of 0.5 mL of 10% (m/v) citric acid. In order to find out the preference for different substrates, 70 nmol of Leu, Ile, Met, Cys, Cys-S-S-Cys, Val, Tyr, Trp, Gln, Thr, Ser, Lys, Arg, Phe, His, and citruline were dissolved in 600 μL of 0.1 M MOPS buffer, pH 7.5. Each solution was incubated with 2 U of purified enzyme at 37 °C and aliquots corresponding to 10 nmol of each amino acid were taken out after 0, 10, 20, 40 and 80 min incubation. The reaction was stopped by 150 μL of 150 mM citrate buffer, pH 2.2 containing 15% (v/v) glycerol and 50 μL of 5% (v/v) TFA. These aliquots were than analyzed in an automatic amino acid analyzer.

3.4. Biochemical characterization

The p*I* and amino acid composition of the purified enzyme were determined as previously described.[31] Treatment with PGNase F under denaturing or nondenaturing conditions: A sample of 15 μg of purified enzyme was dissolved in 20 μL of 50 mM phosphate buffer, pH 7.5, treated with 1 μL of PGNase F (0.08 U/mL) and incubated at 37 °C for 4 h. PAGE and enzymatic assays were subsequently carried out to monitor deglycosylation and activity. Amino acid sequence analysis was performed by a protein microsequencing system. Edman degradation of nonreduced protein was performed in a gas-phase PPSQ-23A Shimadzu sequence equipment using the conditions recommended by the manufacturer. The amino acid sequences were compared to sequences in the GenBank database using the BLAST program.

3.5. Platelet aggregation

The procedure described by Fuly et al.[32] was used in these experiments. Platelet aggregation was measured turbidimetrically using a Whole Blood Lumi-Aggregometer, Chrono-Log Corporation. One effective dose (ED_{50}) was defined as the amount of enzyme that produces 50% platelet aggregation.

3.6. Bactericidal activity

The ability of LAAO to induce bactericidal activity against *E. coli* (ATCC 29648) and *S. aureus* (ATCC 25923) was assayed as previously described.[33,34] The minimum inhibitory concentration (MIC) was visually determined through the macrodilution method using 4×10^5 CFU (colony-forming units)/mL incubated with 24 and 48 μg of purified enzyme for 30 min, 1 h and 24 h in phosphate buffered saline. The minimal bactericidal concentration (MBC) was expressed as the concentration that reduced 99–100% growth.

3.7. Edema-inducing activity

Groups of six Swiss male mice (18–22 g) were injected in the subplantar region with the purified enzyme (50 μg/50 μL). After 0.5, 1 and 3 h, the paw edema was measured with the aid of a low pressure spring caliper (Mitutoyo-Japan).[35]

3.8. Hemorrhagic activity

Samples with different concentrations of purified enzyme in saline (50 μL) were injected intradermically in the back of 20–25 g mice ($n = 6$). After 3 h the animals were killed with ethyl ether, the skins removed and the hemorrhagic halo measured and expressed in mm.[36]

3.9. Statistical analysis

Data are presented as mean values ± SD obtained with recorded number of tested animals. For statistical significance the data were analyzed by Student's unpaired *t*-test at 5% level.

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